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We propose that the expression of Sex Hormone-Binding Globulin (SHBG) by breast cancer cells is biologically regulated and that this SHBG functions to alter the effects of estrogens within the breast cancer cell. The expression of SHBG by breast cancer cells raises the important question of how the local regulation of SHBG synthesis and secretion affects both the sequestration of steroid hormones within the breast, and estrogen induced signal transduction at the cell membrane. The local synthesis of SHBG is consistent with an autocrine/paracrine role for this protein in breast cancer. We have made significant progress setting up our *in vitro* system for studying the effects of SHBG expression on breast cancer growth. We have generated candidate clones, derived from the human MCF-7 breast cancer cell line, that can be induced to either overexpress SHBG or to silence endogenous SHBG expression. These clones, along with a vector control cell line, will serve as vehicles to study the effects of SHBG expression on the regulation of steroid based signaling in human breast cancer.

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Progress report: "Autocrine and Paracrine Control of Breast Cancer Growth by Sex Hormone-Binding Globulin"

INTRODUCTION

We propose that the expression of Sex Hormone-Binding Globulin (SHBG) by breast cancer cells is biologically regulated and that this SHBG functions to alter the effects of estrogens within the breast cancer cell. We have shown that the plasma protein sex hormone-binding globulin (SHBG) not only binds estrogens in plasma, but also is part of an estrogen signal transduction system that starts with a receptor (R_{SHBG}) for SHBG on breast cell membranes rather than the intracellular estrogen receptor (ER). The SHBG-R_{SHBG} complex is activated by an appropriate steroid hormone, such as estradiol (E_2), (forming the new complex, E_2-SHBG-R_{SHBG}), to trigger a second messenger system to produce cAMP within minutes after the steroid binds. We have shown that frozen sections of normal and cancerous breast cells stain with anti-SHBG antibodies and that these same cells contain SHBG mRNA. Further the well-known breast cancer cell line, MCF-7, contains both SHBG mRNA and SHBG protein. The expression of SHBG by breast cancer cells raises the important question of how the local regulation of SHBG synthesis and secretion affects both the sequestration of steroid hormones within the breast, and estrogen induced signal transduction at the cell membrane. The local synthesis of SHBG is consistent with an autocrine/paracrine role for this protein in breast cancer.

We will conduct genetically and pharmacologically based studies to address our hypothesis. The first year of this study has been devoted to the generation of breast cancer cell lines that inducibly overexpress or silence endogenous SHBG expression. These cell lines will be used to investigate the effects of SHBG expression on selective stimulation of either the ER or the SHBG-receptor based pathways. Further, although ER and SHBG both are high affinity binders of E_2, there are other ligands that are specific to each. The detailed pharmacologic approach that we will use will allow us to dissect the influence of estrogens on the two pathways of interest and further to ascertain how each contributes to the growth of breast cancer cells.

BODY

We have made significant progress setting up our *in vitro* system for studying the effects of SHBG expression on breast cancer growth. We cloned the human SHBG cDNA into the inducible expression vector, pINDHyg, in both the sense (pIND/Hygro/SHBGsense) and antisense (pIND/Hygro/SHBGantisense) directions, and confirmed the fidelity of the inserts by sequence analysis. Importantly, we decided that it would be scientifically prudent to establish intermediate MCF-7 and MDA-MB-231 cell lines that have incorporated functional pVgRXR sequences so that all derivative cell lines in these studies would be from identical subclones. In this way, we would minimize artifacts be due to positional effects on pVgRXR genomic insertion points or clonal variance. We concluded that the time cost associated with this modification would be well worth the benefit of generating genetically similar cell lines for this study. We also decided to first

focus on establishment of inducible MCF-7 cell lines to ensure that all our reagents were working properly. We transfected the plasmid, pVgRXR into MCF7 cells, and selected for its stable uptake by growing the transfected cells in the presence of zeocin. Twenty-four candidate zeocin resistant colonies were isolated and expanded as intermediate clonal cell lines. In transient transfection experiments with pIND/Hygro/LacZ, all clones were tested for their ability to induce expression of the LacZ gene following treatment with the inducing agent, Ponasterone A (Pon A). Three intermediate clones, MCF7-pVgRXR4, 12, and 13, demonstrated inducible LacZ activity (β-galactosidase assay). The MCF7-pVgRXR 13 cell line showed no background and had the highest degree of induction; therefore, it was chosen as our intermediate cell line.

The four inducible plasmids, pIND/Hygro/SHBGsense and pIND/Hygro/SHBGantisense, the vector pIND/Hygro, and pIND/Hygro/LacZ (a transfection control) were transfected into MCF7-pVgRXR13 cells. Selection for incorporation of plasmid sequences was in hygromycin B. We isolated and expanded 24 colonies each of the pIND/Hygro/SHBGsense, pIND/Hygro/SHBGantisense, and pIND/Hygro transfectants. We tested the MCF7-pVgRXR13-pIND/Hygro/LacZ pool of cells with Pon A (10uM), and found that approximately 8% of the induced cells turned blue in a β-galactosidase assay, while no uninduced cells from the same pool turned blue. This indicated that, not only was the transfection process successful, but more importantly our overall strategy is feasible. We are currently identifying subclones from the MCF7-pVgRXR13 that we already have isolated, to identify secondary clones that can be induced to overexpress SHBG, silence SHBG expression, and serve as our pIND/Hygro vector control. Because this strategy worked in MCF-7 cells, we have begun parallel experiments in MDA-MB-231 cells. We have performed the first transfection, introducing pVgRXR into MDA-MB-231 cells, and are in the process of isolating candidate intermediate clones for expansion as cell lines. Having a genetically similar background in our SHBG sense, antisense, and vector control cell lines will assist us with the interpretation of later results, and therefore, we think is the wisest way to proceed.

We are also currently expanding on our findings that SHBG is expressed in normal breast tissue by designing pilot experiments to investigate SHBG expression in breast tumor tissue. We have obtained paired normal and tumor tissues from the Columbia University tumor tissue bank, and we plan to use these specimens in immunohistochemistry and in situ hybridization (if the tumor RNA is viable) experiments. We have also begun the process of generating rabbit polyclonal antibodies against peptide sequences specific for $SHBG_L$ and $SHBG_T$, and plan to evaluate these antibodies in the upcoming year for use in immunohistochemistry and Western blotting experiments. If proven effective, these antibodies will allow us to investigate isoform specific expression of SHBG. Finally, we have previously demonstrated that SHBG localizes at the membranes of MCF-7 and MDA-MB-231 cells, consistent with its binding to R_{SHBG}. We are in the midst of experiments to evaluate whether incubation of these cells with specific steroids known to be noncompetitive antagonists of R_{SHBG} binding, will abolish SHBG localization at the membrane, consistent with our model for SHBG signaling.

KEY RESEARCH ACCOMPLISHMENTS

Task 1 Generate breast cancer cell lines that stably express sex hormone-binding globulin (SHBG) in a regulatable fashion.

- Develop plasmid constructs encoding the full length human SHBG cDNA, in the sense and antisense orientations (Months 1-2)—**completed.**
- Generate stably transfected MCF-7 and MDA-MB-231 cell lines containing a regulatable promoter upstream of: 1) the sense (MCF-7_{SHBG}; MDA-MB-231 $_{SHBG}$) construct, 2) the antisense (MCF-7_{noSHBG}; MDA-MB-231 $_{noSHBG}$) construct, and 3) the vector (MCF-7_{vector}; MDA-MB-231 $_{vector}$) as a control.
- generation and evaluation of intermediate MCF7 clones that have been stably transfected with the plasmid, pVgRXR, encoding the components of the functional transactivator (Months 3-8) —**completed.**
- b2: generation and evaluation of secondary transfectant clones that can be induced to overexpress or silence SHBG, and the corresponding vector control cell line (Months 9-13) —**in the latter stages of completion.**

Other research accomplishments not included in, but related to our initial study:

- Paired normal and tumor tissues obtained for evaluation of SHBG expression in breast cancer specimens.
- Rabbits inoculated with peptides specific for either $SHBG_L$ or $SHBG_T$, to generate isoform-specific polyclonal antisera.
- Conditions established for studying the effects, in MCF-7 and MDA-MB-231 cells, of noncompetitive steroid agonists of R_{SHBG} binding on the membrane localization of SHBG.

REPORTABLE OUTCOMES: Not applicable at this early stage

CONCLUSIONS: Not applicable at this early stage

REFERENCES: Not applicable at this early stage

APPENDICES: Not applicable at this early stage